{Exhibit 61}

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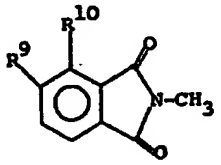
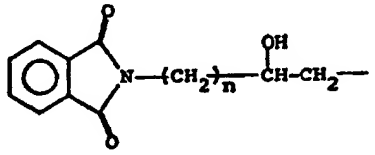
(74) Agents J. A. Kemp & Co.

(54) Intermediates useful in th synthesis of chemiluminescent-labeled conjugates for use in specific binding assays

(57) Novel compounds of the formula:



wherein one of R^9 and R^{10} is hydrogen and the other is $—NR^{11}R^{12}$; R^{11} is hydrogen or straight chain alkyl containing 1—4 carbon atoms and R^{12} is



wherein n=1 to 3, are intermediates in the synthesis of chemiluminescent-labeled conjugates useful in specific binding assays.

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SPECIFICATION

Intermediates useful in the synthesis of chemiluminescent-lab led conjugates for use in sp cific binding assays

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

This invention relates to novel intermediates useful in the synthesis of chemiluminescent-labeled conjugates for use in specific binding assays for a ligand, such as an antigen, hapten or antibody, in a liquid medium such as a body fluid. This Application is a divisional of our Application No. 7923660 filed July 6th 1979. (Serial No. 2026690).

The desirability of a convenient, reliable, and non-hazardous means for detecting the presence of low concentrations of substances in liquids is self-evident. This is particularly true in the field of clinical chemistry where constituents of body fluids which may appear in concentrations as low as 10^{-11} molar are known to be of pathological significance. The difficulty of detecting such low concentrations is compounded in the field of clinical chemistry where sample size is usually quite limited.

Classically, substances have been detected in liquids based on a reaction scheme wherein the substance to be detected is a necessary reactant. The presence of unknown is indicated by the appearance of a reaction product or the disappearance of a known reactant. In certain instances, such an assay method may be quantitative, based on a measurement of either the rate of appearance of product or disappearance of reactant or measurement of the aggregate amount of product produced or reactant consumed in attaining equilibrium. Each assay reaction system is necessarily either limited to use in the detection of only a small group of substances or is nonspecific.

The search for assay systems which are highly specific yet adaptable to the detection of a wide range of substances has evolved the radioimmunoassay. In this system a known amount of a radiolabeled form of the substance to be detected is allowed to compete with the unknown for a limited quantity of antibody specific for the unknown. The amount of the labeled form that becomes bound to antibody varies inversely with the level of unknown present. Inherent in the radioimmunoassay technique is the need to separate the labeled form of substance to be detected which becomes bound to antibody from that which does not become so bound. While various ways of accomplishing the required separation have been developed, as exemplified in U.S. Patents Nos. 3,505,019; 3,555,143; 3,646,346; 3,720,760; and 3,793,445, all require at least one separate manipulative step, such as filtering, centrifuging, or washing, to insure efficient separation of the bound-labeled form from the unbound-labeled form. The elimination of the separation step would greatly simplify the assay and render it more useful to the clinical laboratory.

The use of radioactive materials in immunoassays has been eliminated to some degree by the use of enzyme-tagged materials in place of radiolabels. As exemplified by U.S. Patents Nos. 3,654,090 and 3,791,932, the manipulative steps necessary for carrying out the enzyme-tagged immunoassays are for the most part the same as those required in radioimmunoassays and include the cumbersome separation step. An additional disadvantage of using enzyme-tagged materials is that each enzyme used as a tag must be individually chemically modified for use in the formation of the tagged conjugate. The use of other tagging materials has been suggested, such as the use of coenzymes or viruses, *Nature 219*:186(1968) and the use of fluorescent labels, French Patent No. 2,217,350 corresponding to U.S. Patent No. 3,880,934.

2. BRIEF DESCRIPTION OF THE PRIOR ART

While these radiolabeled and enzyme-tagged immunoassays may undergo future improvement in terms of expansion of the range of substances detectable thereby or of simplification of the procedure, by their nature they will always require some type of separation step. Recently, a different approach was disclosed which does not require a separation step and therefore has been referred to as a homogeneous system, in contrast to a heterogeneous system in which separation is essential. U.S. Patent No. 3,817,837 discloses a competitive binding assay method involving the steps of combining the liquid to be assayed with a soluble complex consisting of an enzyme as a labeling substance covalently bound to the ligand to be detected and with a soluble receptor, usually an antibody, for the ligand; and analyzing for the effect of the liquid to be assayed on the enzymatic activity of the enzyme in the complex.

While this method has the advantage of not requiring a separation step because reaction between the enzyme-bound-ligand complex and the rec ptor results in inhibition of the enzymatic activity of the enzyme in the complex, the method nonetheless is severely restricted in its ability to be adapted to widely varied assay requirements. For instance, it is clearly essential that in the fabrication of the enzyme-bound-ligand complex, the substance or ligand to be detected must be coupled to the enzyme in a carefully controlled manner so that the coupling site is close to the enzymatically active site on th enzyme. This is required in order that upon reaction between the complexed ligand and the receptor, the enzymatically active site is blocked. Enzymes vary greatly in their size, ranging in molecular weight from about 10,000 to 1,000,000. Thus, for a receptor in the form of an antibody having a molecular weight

of between 150,000 and 300,000 to be capable of physically blocking the active site on an average enzyme of 500,000 molecular weight or greater, the coupling site must be precisely controlled. Due to the complex chemical structure of enzymes, precise control of such chemical linkage is indeed difficult, and one would expect that even upon screening a wide variety of enzymes only a small number would be found to be of use in this homogeneous assay system.

Moreover, it is critical for the purpose of obtaining quantitative test results to precisely control the ratio of the number of enzymes to the number of ligands in each enzyme-bound-ligand complex. Here also, the complex peptide structure of enzymes makes such control difficult. It would again be expected that only a small number of enzymes would have suitable molecular structure to ensure necessary control of the ligand/enzyme ratio.

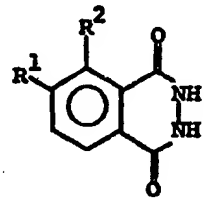
The prior art homogeneous assay method is stated to involve an enzyme amplification and thus to be highly sensitive. However, since the labeling substance, namely the enzyme, is itself the limiting factor determining the sensitivity of the prior art assay method, the versatility of the method is severely restricted. The sensitivity is clearly limited to the catalytic activity of the particular enzyme in the enzyme-bound-ligand conjugate. The versatility of the prior art method is therefore restricted not only by the coupling requirements for formation of a useful conjugate but also by the dependence of the sensitivity of the assay that employs such conjugate on the activity of the particular conjugated enzyme.

An additional disadvantage of the prior art homogeneous assay method arises in its application to the testing of biological fluids, such as urine and serum. It is to be expected that significant amounts of the enzyme species comprised in the enzyme-bound-ligand conjugate may appear in the fluid sample to be tested thereby creating an uncontrollable background activity which would severely affect the accuracy of the assay method. Therefore, in order to form an assay system that is useable in testing biological fluids of humans or animals, exotic enzymes not endogenous to such fluids must be selected for use in forming the enzyme-bound-ligand conjugate with the result that the versatility of the assay method is even further restricted.

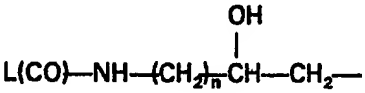
The present invention provides novel intermediates useful in the synthesis of chemiluminescent-labeled conjugates for use in specific binding assays as described in our aforesaid Application No. 7923660. (Serial No. 2026690).

In our Application No. 7923660 out of which this Application has been divided, we have described and claimed a highly convenient, versatile, and sensitive homogeneous specific binding assay method and system based on the use of, as labeling substance, a reactant in a chemiluminescent reaction. The method is based, in part, on the fact that the reaction between a ligand and a specific binding partner thereof to one of which the said reactant is coupled in the form of a conjugate, alters the activity of the reactant in the chemiluminescent reaction, which reaction thus serves as means for monitoring the specific binding reaction. Various manipulative schemes involving various test compositions and devices may be employed in performing the method and are described in the aforesaid Application.

Particularly useful chemiluminescent-labeled conjugates described in out aforesaid Application are those of the formula:



wherein one of R^1 and R^2, preferably R^2, is hydrogen and the other is $—NR^3R^4$; R^3 is hydrogen or straight chain alkyl containing 1—4 carbon atoms, preferably ethyl, and R^4 is



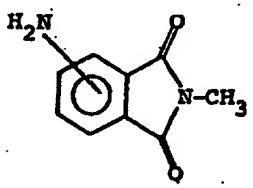
wherein n = 1 to 3, preferably 1, and L(CO)— is a specifically bindable ligand, or a binding analog thereof, bound through an amide bond.

The specifically bindable ligand or analog thereof in the present labeled conjugates, in terms of its chemical nature, usually is a protein, polypeptide, peptide, carbohydrate, glycoprotein, steroid, or other organic molecule for which a specific binding partner is obtainable. In functional terms, the ligand will usually be an antigen or an antibody thereto; a hapten or an antibody thereto; or a hormone, vitamin, or drug, or a receptor or binding substance therefor. Most commonly, the ligand is an immunologically-

active polypeptide or protein of molecular weight between 1,000 and 4,000,000 such as an antigenic polypeptide or protein or an antibody; or is a hapten of molecular weight between 100 and 1,500.

The aforesaid labeled conjugates are prepared usually by forming a peptide or amide couple between (1) an amino derivative of a chemiluminescent aminophthalhydrazide (e.g., luminol or isoluminol) and (2) either the ligand, where such contains a carboxylic acid function, or a binding analog of the ligand (e.g., a derivative of the ligand) which analog contains the desired carboxylic acid function. Such condensation reactions can be accomplished by reacting the amino derivative of the label directly with the carboxylic acid-containing ligand or ligand analog using conventional peptide condensation reactions such as the carbodiimide reaction [*Science 144:*1344 (1964)], the mixed anhydride reaction [Erlanger *et al, Methods In Immunology and Immuno-chemistry,* ed. Williams and Chase, Academic Press (New York 1967) p. 149]; and the acid azide and active ester reactions [Kopple, *Peptides and Amino Acids,* W.A. Benjamin, Inc. (New York 1966)]. See also for a general review *Clin. Chem. 22:* 726 (1976).

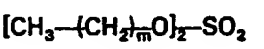
Preferably however the aforesaid chemiluminescent-labeled conjugates are prepared according to the following general synthetic sequence:



(I)

The starting material for the synthesis is 3- or 4-amino-N-methylphthalimide (I) with the 3-amino compound [Wang *et al, JACS 72:*4887 (1950) and Flitsch, *Chem. Ber. 94:* 2494 (1961)] to be used to prepare luminol based labeled-conjugates and the 4-amino compound [Flitsch, *Chem. Ber. 94:* 2494 (1961)] to be used to prepare isoluminol based labeled-conjugates.

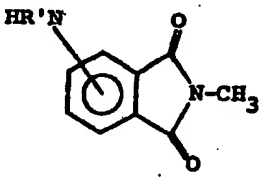
Alkylation of the amino group in the phthalimide (I) is obtained by reaction with a dialkyl sulfate (II) [Rodd, *Chemistry of Carbon Compounds,* vol. 1, Elsevier Publ. Co. (New York 1951) p. 337]



(II)

$m = 0—3$

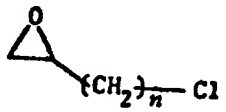
to yield the N-alkylated derivative (III)



(III)

wherein R' is straight chain alkyl containing 1—4 carbon atoms.

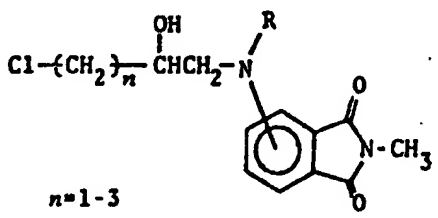
Treatment of the phthalimide (*I*) or its N-alkylated derivative (*III*) with a chloro-epoxide (*IV*) [available from Aldrich Chemical Co., Milwaukee, Wisconsin USA, or see Paul *et al, Bull. Soc. Chim. Fr.* 197 (1948) or Reppe *et al, Justus Liebig's Annalen der Chemie 596:* 80—158 (1955)]



(IV)

n=1-3

produces the chloro-intermediate (*V*)

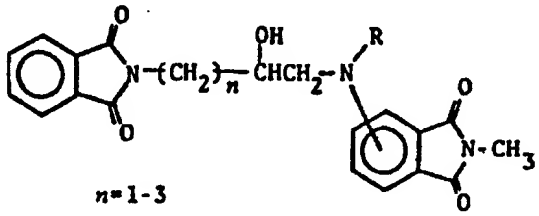


(V)

n=1-3

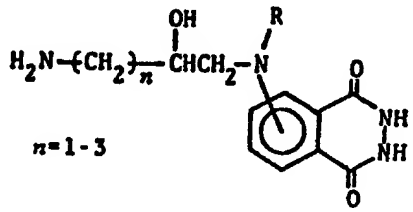
wherein R is hydrogen or straignt chain alkyl containing 1—4 carbon atoms.

Reaction of the chloro-intermediate (*V*) with potassium phthalimide produces the *bis*-phthalimide intermediat (*VI*)



(*VI*)

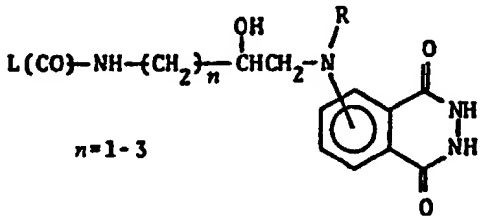
wherein R is the same as defined above, which upon treatment with hydrazine produces the amino-hydrazide (*VII*)



(*VII*)

wherein R again is the same as defined above.

Condensation of the amino-hydrazide (*VII*) with (a) the ligand to be labeled, where such contains a carboxylic acid function, (b) a binding analog of the ligand, such analog being a carboxylic acid derivative of the ligand, or (c) the ligand or an appropriate derivative of the ligand in the presence of a bifunctional coupling agent, produces the chemiluminescent-labeled conjugates (*VIII*)

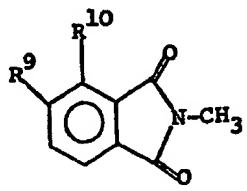


(*VIII*)

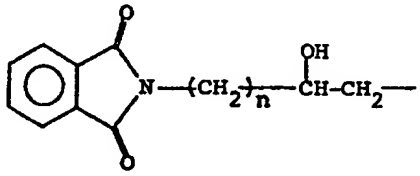
wherein R is the same as defined above and L(CO)— represents the specifically bindable ligand, or a binding analog thereof formed by derivation of the ligand and/or insertion of a bridge by a bifunctional coupling agent), bound through an amide bond.

Other variations of labeled conjugates based on the above-described synthetic scheme are clearly evident. In particular, various ring-substituted amino-N-methylphthalimides may be used as starting material to produce ring-substituted labeled conjugates possessing substantially the same qualitative properties as the conjugates prepared according to the above-described scheme. Such conjugates will be recognized as equivalents and are exemplified by the addition of one, two or more simple substituents to an available aromatic ring site, such substituents including without limitation, alkyl, e.g., methyl, ethyl and butyl; halo, e.g., chloro and bromo; nitro; hydroxyl; alkoxy, e.g., methoxy and ethoxy, and so forth.

As illustrated in the above-described synthetic scheme, the novel intermediate compounds of the present invention produced in the course of preparing the chemiluminescent-labeled conjugates have the following general formula:



wherein one of R^9 and R^{10}, preferably R^{10}, is hydrogen and the other is $—NR^{11}R^{12}$; R^{11} is hydrogen or straight chain alkyl containing 1—4 carbon at ms, preferably ethyl, and R^{12} is



wherein n = 1 to 3, preferably 1.

As stated hereinabove, the ligand which is comprised in the labeled conjugate or whose binding analog is comprised in the labeled conjugate is in most circumstances an immunologically-active polypeptide or protein of molecular weight between 1,000 and 4,000,000 such as an antigenic polypeptide or protein or an antibody; or is a hapten or molecular weight between 100 and 1,500. Following will now be presented various methods for coupling such ligands or analogs thereof to the amino-derivative (VII) of the label through an amide bond.

Polypeptides and Proteins

Representative of specifically bindable protein ligands are antibodies in general, particularly those of the IgG, IgE, IgM and IgA classes, for example hepatitis B antibodies; and antigenic proteins such as insulin, chorionic gonadotropin (e.g., HCG), carcinoembryonic antigen (CEA), myoglobin, hemoglobin, follicle stimulating hormone, human growth hormone, thyroid stimulating hormone, (TSH), human placental lactogen, thyroxine binding globulin (TBG), intrinsic factor, transcobalamin, enzymes such as alkaline phosphatase and lactic dehydrogenase, and hepatitis-associated antigens such as hepatitis B surface antigen (HB_sAg), hepatitis e antigen (HB_eAg) and hepatitis core antigen (HB_cAg). Representative of polypeptide ligands are angiotensin I and II, C-peptide, oxytocin, vasopressin, neurophysin, gastrin, secretin, and glucagon.

Since, as peptides, ligands of this general category possess numerous available carboxylic acid and amino groups, coupling to the amino-derivative of the chemiluminescent label can proceed according to conventional peptide condensation reactions such the carbodiimide reaction, the mixed anhydride reaction, and so forth as described hereinabove, or by the use of conventional bifunctional reagents capable of coupling carboxylic acid or amino functions to the amino group in the label derivative as likewise described above. General references concerning the coupling of proteins to primary amines or carboxylic acids are mentioned in detail above.

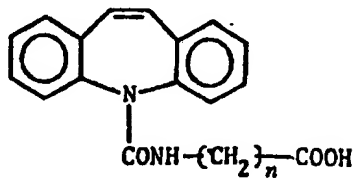
Haptens

Haptens, as a class offer a wide variety of organic substances which evoke an immunochemical response in a host animal only when injected in the form of an immunogen conjugate comprising the hapten coupled to a carrier molecule, almost always a protein such as albumin. The coupling reactions for forming the immunogen conjugates are well developed in the art and in general comprise the coupling of a carboxylic acid ligand or a carboxylic acid derivative of the ligand to available amino groups on the protein carrier by formation of an amide bond. Such well known coupling reactions are directly analogous to the present formation of labeled conjugates by coupling carboxylic acid ligands or binding analogs to the amino-derivative of the chemiluminescent label.

Hapten ligands which themselves contain carboxylic acid functions, and which thereby can be coupled directly to the amino-derivative of the label, include the iodothyronine hormones such as thyroxine and liothyronine, as well as other materials such as biotin, valproic acid, folic acid and certain prostaglandins. Following are representative synthetic routes for preparing carboxylic acid binding analogs of hapten ligands which themselves do not contain an available carboxylic acid function whereby such analogs can be coupled to the amino-derivative of the label by the aforementioned peptide condensation reactions or bifunctional coupling agent reactions (in the structural formulae below, *n* represents an integer, usually 1 through 6).

Carbamazepine

Dibenz[b,f]azepine is treated sequentially with phosgene, an ω-aminoalkanol, and Jones reagent (chromium trioxide in sulfuric acid) according to the meth d of Singh, U.S. Pat. No. 4,058,511 to yield the following series of carboxylic acids:

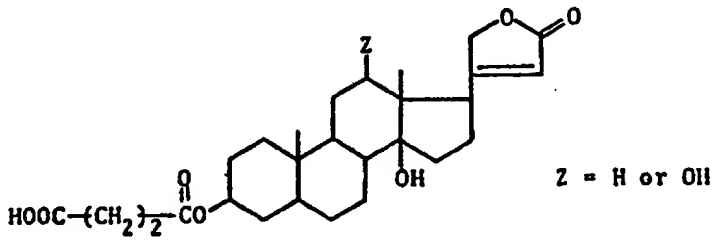


Quinidine

Following the method of Cook *et al, Pharmacologist 17:* 219 (1975), quinidine is demethylated and treated with 5-bromovalerate followed by acid hydrolysis to yield a suitable carboxylic acid derivative.

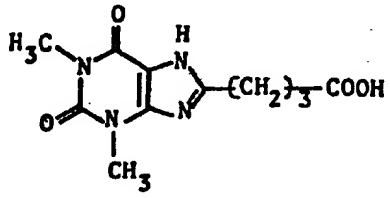
Digoxin and Digitoxin

The aglycone of the cardiac glycoside is treated with succinic anhydride and pyridine according to the method of Oliver *et al, J. Clin. Invest. 47:* 1035 (1968) to yield the following:



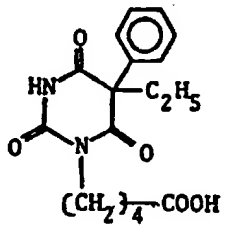
Theophylline

Following the method of Cook *et al, Res. Comm. Chem. Path. Pharm. 13:* 497 (1976), 4,5-diamino-1,3-dimethylpyrimidine-2,6-dione is heated with glutaric anhydride to yield the following:

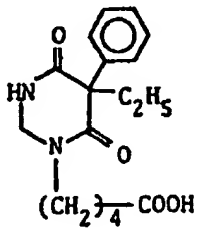


Phenobarbital and Primidone

Sodium phenobarbital is heated with methyl 5-bromovalerate and the product hydrolyzed to the corresponding acid derivative of phenobarbital [Cook *et al, Quantitative Analytic Studies in Epilepsy,* ed. Kelleway and Peterson, Raven Press (New York 1976) pp. 39—58]:

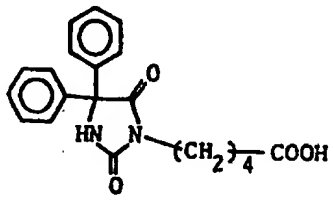


To obtain the acid derivative of primidone following the same Cook *et al* reference method, 2-thiophenobarbital is alkylated, hydrolyzed, and the product treated with Raney nickel to yield:



Diphenylhydantoin

Following the method of Cook *et al, Res. Comm. Chem. Path. Pharm. 5:* 767 (1973), sodium diphenylhydantoin is reacted with methyl 5-bromovalerate followed by acid hydrolysis to yield the following:

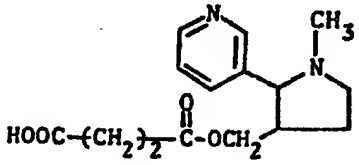


Morphine

Morphine free base is treated with sodium β-chloroacetate according to the method of Spector *et al, Science 168:* 1347 (1970) to yield a suitable carboxylic acid derivative.

Nicotine

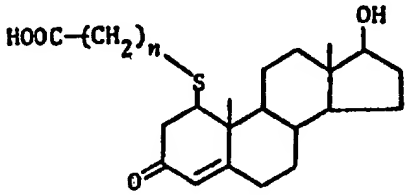
According to the method of Langone *et al, Biochem. 12(24):* 5025 (1973), *trans*-hydroxymethylnicotine and succinic anhydride are reacted to yield the following:



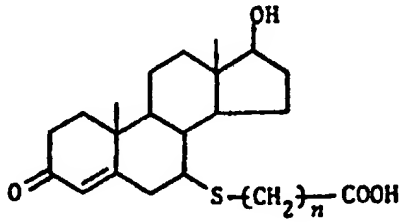
Androgens

Suitable carboxylic acid derivatives of testosterone and androstenedione linked through either the 1- or 7-position on the steroid nucleus are prepared according to the method of Bauminger *et al, J. Steroid Biochem. 5:* 739 (1974). Following are representative testosterone derivatives:

1-position

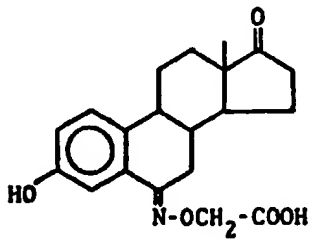


7-position



Estrogens

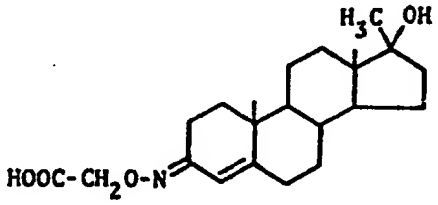
Suitable carboxylic acid derivatives of estrogens, e.g., estrone, stradiol and estriol, are prepared according to the method of Bauminger *et al, supra,* as represented by the following estrone derivative:



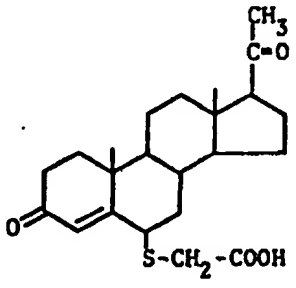
Progesterones

Suitable carboxylic acid derivatives of progesterone and its metabolites linked through any of the 3-, 6- or 7-positions on the steroid nucleus are prepared according to the method of Bauminger *et al, supra,* as represented by the following progesterone derivatives:

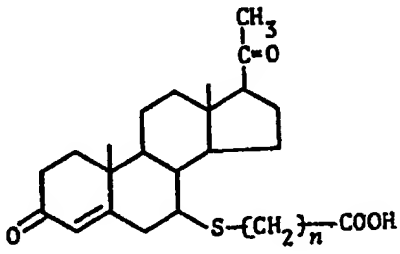
3-position



6-position



7-position



The methods described above are but examples of the many known techniques for forming suitable carboxylic acid derivatives of haptens of analytical interest. The principal derivation techniques are discussed in *Clin. Chem. 22:* 726 (1976) and include esterification of a primary alcohol with succinic anhydride [Abraham and Grover, *Principles of Competitive Protein-Binding Assays,* ed. Odell and Daughaday, J. B. Lippincott Co. (Philadelphia 1971) pp. 140—157], formation of an oxime from reaction of a ketone group with carboxylmethyl hydroxylamine [*J. Biol. Chem. 234:* 1090 (1959)], introduction of a carboxyl group into a phenolic residue using chloroacetate [*Science 168:* 1347 (1970)], and coupling to diazotized *p*-aminobenzoic acid in the manner described in *J. Biol. Chem. 235:* 1051 (1960).

The present invention will now be illustrated, but is not intended to be limited, by the following Examples:

Biotin Conjugate

A. Preparation of the Labeled Conjugate

The reaction sequence for this sythesis is described and shown schematically in *Anal. Chem. 48:* 1933 (1976).

4-(3-Chloro-2-hydroxypropylamino)-N-methylphthalimide.

Twenty-five grams (g) (0.142 mole) 4-amino-N-methylphthalimide [Flitsch, *Chem. Ber. 94:* 2494 (1961)] and 20.7 g (0.21 mole) 1-chloro-2,3-epoxypropane were added to 150 ml 2,2,2-trifluoroethanol and the reaction mixture was heated to reflux with stirring for 48 hours. Seventy to eighty ml of 2,2,2-trifluoroethanol was removed by distillation and a heavy yellow precipitate formed when the remaining solution cooled to room temperature. This precipitate was triturated with ethyl acetate, collected by filtration and dried to give 29.5 g (77% yield) of the desired phthalimide intermediate m.p. 136—138.5°C.

Analysis: Calculated for $C_{12}H_{13}ClN_2O_3$: C, 53.64; H, 4.88; N, 10.45
Found: C, 53.87; H, 4.85; N, 10.81

4-[3-(N-Phthalamido)-2-hydroxypropylamino]-N-methylphthalimide.

The phthalimide intermediate prepared above (13.5 g, 0.05 mole) and 15.7 g (0.085 mole) potassium phthalimide were heated to reflux with stirring in 150 ml dimethylformamide for 24 hours. The dimethylformamide was removed and the residue was washed with water and filtered. The yellow filter cake was recrystallized from acetic acid-water to give 12.8 g (67% yield) of the *bis*-phthalimide intermediate, m.p. 247—248.5°C.

Analysis: Calculated for $C_{20}H_{17}N_3O_6$: C, 63.32; H, 4.52; N, 11.08
Found: C, 63.16; H, 4.38; N, 10.93

6-(3-Amino-2-hydroxypropylamino)-2,3-dihydrophthalazine-1,4-dione.

The *bis*-phthalimide intermediate from above (5.0 g, 13.2 mmole), 90 ml absolute ethanol and 35 ml 95% hydrazine were refluxed with stirring for 4 hourrs. The solvent was removed under a vacuum and the resulting solid was dried for 24 hours under vacuum at 120°C. This material was stirred for 1 hour with 70 ml of 0.1 N hydrochloric acid. The insoluble material was removed by filtration and the filtrate was adjusted to pH 6.5 with saturated sodium bicarbonate. The white precipitate which formed was collected by filtration and dried to give 2.2 g of the product (67% yield). After recrystallization from water, the compound decomposed at 273°C.

Analysis: Calculated for $C_{11}H_{14}N_2O_3$: C, 52.79; H, 5.64; N, 22.39
Found: C, 52.73; H, 5.72; N, 22.54

The efficiency of the amino-derivative (i.e., the label derivative) in a chemiluminescent reaction and the detection limit of such derivative were determined as follows.

In determining efficiency, the label derivative and luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) were oxidized individually at several levels in the picomolar range and related to the peak light intensities generated by a graph plot. Linear portions of the resulting curves allowed calculation of change in peak light intensity per unit concentration for the label derivative and for luminol. Efficiency of the label derivative was expressed as a percentage of the slope produced with luminol.

Reaction mixtures (150 μl) of the following composition were assembled in 6 x 50 mm test tubes mounted in a Dupont 760 Luminescence Biometer (E.I. duPont de Nemours and Co., Wilmington, Delaware USA) with a sensitivity setting of 820: 50 mM sodium hydroxide, 0.07 μM hematin (Sigma Chemical Co., St. Louis, Missouri USA) and either the amino-derivative or luminol at varying concentrations in the picomolar (pM) range (diluted with H_2O from a 1 mM stock soslution in 0.1 M sodium carbonate, pH 10.5). Each mixture was incubated 10 minutes at room temperature and 10 μl of 90 mM hydrogen peroxide was added to initiate the chemiluminescent reaction. Peak light intensity values were recorded from the instrument readings. All reactions were performed in triplicate and averaged. The efficiency of the label derivative was found to be 10%.

Detection limit was defined as the concentration of the label derivative that produced a peak light intensity one and a half times the background chemiluminescence in the reaction mixture. The detection limit for the label derivative was found to be 20 pM.

The N-ethylated derivative, 6-[N-(3-amino-2-hydroxypropyl)-N-ethylamino]-2,3-dihydrophthalazine-1,4-dione, of the above-described amino-derivative was also prepared by treating 4-amino-N-methylphthalimide with diethyl sulfate under reflux in 2,2,2-trifluoroethanol and then following the same synthesis as described above to convert the N-ethylated intermediate through the phthalimide and *bis*-phthalimide intermediate stages t the N-ethylated amino-derivative. The efficiency of this compound in the hematin catalyzed chemiluminescent reaction was found to be 46% and the

detection limit 5 pM.

6-(3-Biotinylamino-2-hydroxypropylamino)-2,3-dihydrophthalazine-1,4-dione [biotin-isoluminol conjugate].

Biotin (0.29 g, 1.2 mmole) and 0.17 ml triethylamine were dissolved in 20 ml dry dimethylformamide under anhydrous conditions and cooled to −10°C. A solution of 0.141 ml thyl chloroformate in 2.86 ml ether was added slowly and the reaction was stirred for 30 minutes. A precipitate which formed was separated by filtration. A suspension consisting of 600 mg (2.4 mmole) of the amino-derivative intermediate from above, 20 ml dry dimethylformamide and 1 ml dry pyridine was added to the filtrate quickly. This mixture was stirred at −10°C for 30 minutes and then at room temperature overnight. During this period a solution was obtained. The dimethylformamide was removed by distillation at 60°C and 0.10 mm Hg pressure. The oily residue was stirred with 50 ml of 0.1 N hydrochloric acid for 1 hour. A white solid which formed was filtered and washed with 0.1 N hydrochloric acid and then water. After drying under a vacuum at room temperature overnight, 0.55 g (97% yield) of the labeled conjugate was obtained, m.p. 170—3°C.

Analysis: Calculated for $C_{21}H_{28}N_6O_6S$: C, 52.92; H, 5.92; N, 17.64
Found: C, 51.69; H, 5.90; N, 17.63

B. Binding Assays for Biotin and Avidin Using Enzyme Catalyzed Monitoring Reaction

The chemiluminescent reacton system used in this example was based on the following reaction:

$$\text{biotin-isoluminol} + H_2O_2 \xrightarrow{\text{lactoperoxidase}} \text{biotin-aminophthalate} + N_2 + h\nu$$

Nine specific binding reaction mixtures were prepared, each having a total volume of 140 μl and each containing 0.1 M tris-(hydroxymethyl)-aminomethane hydrochloride buffer (Tris-HCl) at pH 7.4 and biotin, biotin-isoluminol labeled conjugate (prepared as above), and avidin (added last) in the concentrations indicated in Table 1. After 5 minute incubation at 25°C, 10 μl 0.1 M Tris-HCl buffer at pH 7.4 containing 20 units/ml lactoperoxidase [Sigma Chemical Co., St. Louis, Missouri USA; assayed as described in *Methods in Enzymology* XVIIA, (1970) p. 653-Assay 2] was added to each reaction mixture. After incubation at 25°C for 2 additional minutes, 10 μl of 0.95 mM hydrogen peroxide in 10 mM Tris-HCl buffer at pH 7.4 was injected into each reaction mixture and the peak light intensity produced in each was measured using the Dupont Model 760 Bioluminescence Photometer. The results appear in Table 1.

TABLE 1

reaction mixture	concentration of biotin (μM)	concentration of biotin-isoluminol conjugate (nM)	concentration of avidin (units/ml)	peak light intensity
1	—	—	—	0.8
2	—	—	0.14	0.9
3	—	84	—	1.9
4	—	84	0.14	25.3
5	4	—	—	0.8
6	4	84	—	2.2
7	4	—	0.14	0.9
8	4	84	0.14	6.1
9	1.3	84	0.14	10.4

Reactions 1,2,5 and 7 were controls and show that in the absence of biotin-isoluminol conjugate, only a low background amount of light was measured. The result of reactions 3 and 6 indicate the

biotin-isoluminol conjugate was active in the chemiluminescent reaction and that the presence of free biotin had no significant effect on such activity. The result of reaction 4 shows that in the presence of avidin, a binder for biotin, the activity of the biotin-isoluminol conjugate increased. This result is rather unexpected since one would anticipate that binding of avidin to the conjugate should limit the availability of the isoluminol moiety for th chemiluminesc nt reaction. The reason for the observed enhancement of light-production is not understood. A comparison of the results of reactions 4,8, and 9 demonstrate that the enhancement of light production is decreased inversely with the amount f free biotin present.

This example demonstrates that the ligands avidin and biotin can be determined using the present labeled conjugates and that according to the present invention the effect of binding between the labeling substance in the conjugate and a corresponding binding partner may be an enhancement, rather than inhibition, of the activity of the labeling substance.

A further experiment was conducted using the same lactoperoxidase-catalyzed monitoring reaction.

Six specific binding reaction mixtures were prepared, each having a total volume of 140 μl and each containing 0.1 M Tris-HCl buffer at pH 7.4, 84 nM biotin-luminol conjugate (prepared as above), biotin at the concentrations indicated in Table 2, and 0.035 units/ml avidin (added last). After a 5 minute incubation at 25°C, 10 μl of lactoperoxidase (20 units/ml) were added to each reaction mixture.

After an additional 2 minute incubation, 10 μl 0.95 mM hydrogen peroxide in 10 μM tris-HCl buffer at pH 7.4 was injected into each reaction mixture and the peak light intensity produced in each was measured as in the previous experiment. The results appear in Table 2.

TABLE 2

reaction mixture	concentration of biotin (nM)	peak light intensity
1	0	23.5
2	67	21.1
3	134	15.5
4	200	12.6
5	268	12.3
6	400	8.1

It was thus demonstrated that the magnitude of the peak light intensity produced by the chemiluminescent reaction system was an inverse function of the amount of biotin present in the specific binding reaction mixture. The present invention therefore provides labeled conjugates useful for determining the presence of ligands in a liquid medium.

C. Binding Assay for Biotin Using Non-Enzymatic Monitoring Reaction.

The chemiluminescent reaction system used in this example was based on the following reaction:

$$\text{biotin-isoluminol} + KO_2 \longrightarrow \text{biotin-aminophthalate} + N_2 + h\nu$$

Sixt en specific binding reaction mixtures were prepared, each having a total volume of 150 μl and each containing 0.1 M Tris-HCl at pH 8.0, 42 nM biotin-isoluminol conjugate (prepared as above), biotin at the concentrations indicated in Table 3, and 0.12 units/ml avidin (added last). After incubation at 25°C f r 5 minutes, 10 μl of dimethylformamide containing 0.15 M potassium superoxide (KO_2) (Alpha Products, Beverly, Massachusetts USA) and 0.10 M 1,4,7,10,13,16-hexaoxacylcooctadecane (Aldrich Chemical Co., Milwaukee, Wisconsin USA) were injected into each reaction mixture and the peak light intensity produced in each was measured as in the previous experiments. The results appear in Table 3.

TABLE 3

reaction mixture	concentration of biotin (nM)	peak light intensity
1	0	38.5
2	13	38.5
3	27	34.3
4	40	36.1
5	53	35.2
6	67	36.2
7	101	34.0
8	133	31.7
9	166	29.1
10	200	24.2
11	267	22.8
12	333	20.5
13	400	13.4
14	534	8.6
15	667	8.3
16	800	7.0

It was demonstrated that the magnitude of the peak light intensity produced by the chemiluminescent reaction system was an iverse function of the amount of biotin present in the specific binding reaction mixture. The present invention therefore provides labeled conjugates useful for determining the presence of ligands in a liquid medium.

Thyroxine Conjugates

A. Preparation of Labeled Conjugates

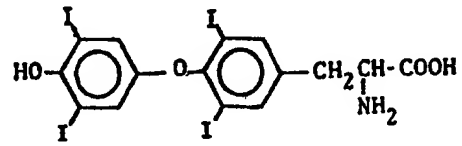
Following are descriptions of the preparation of the labeled thyroxine conjugates 6-N-[2-hydroxy-3-(thyroxinylamido)propyl]amino-2,3-dihydrophthalazine-1,4-dione and 6-[N-ethyl-N-[2-hydroxy-3-(thyroxinylamido)propyl]amino}-2,3-dihydrophthalazine-1,4-dione. The reaction sequences for these syntheses are outlined in Tables 4 and 5.

N-Trifluoroacetylthyroxine (2)

A solution of 20 grams (g) [25.6 millimole (mmol)] of L-thyroxine (*1*) (Sigma Chemical Co., St. Louis, Missouri USA) in 240 milliliters (ml) of ethyl acetate containing 46 ml of trifluoroacetic acid and 7.6 ml of trifluoroacetic anhydride was stirred at 0°C for one hour. Upon adding 200 ml of water (H_2O), a suspension formed that was saturated with sodium chloride. The organic phase was separated, washed with saturated aqueous sodium chloride solution, dried ov r anhydrous magnesium sulfat , filtered and evaporated. When dry, the crystalline residue amounted to 21.3 g of the N-protected thyroxine derivativ (*2*). A sample was recrystallized from ether-pentane to give fine white crystals, melting point (m.p.) 233—235°C (decomposed).

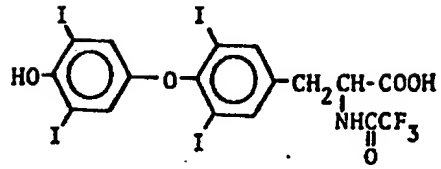
Analysis: Calculated for $C_{17}H_{10}F_3I_4NO_5$: C, 23.39; H, 1.15; N, 1.60
Found: C, 23.23; H, 1.12; N, 1.59
Infrared Spectrum (KCl): 1700 cm^{-1} (carbonyl)
Optical Rotation $[\alpha]_D^{25} = -14.97°$ (c 1.0, dimethylsulfoxide)

TABLE 4



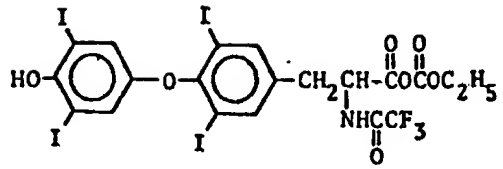
(1)

CF_3COOH



(2)

$ClCOOC_2H_5 - (C_2H_5)_3N$



(3)

TABLE 5

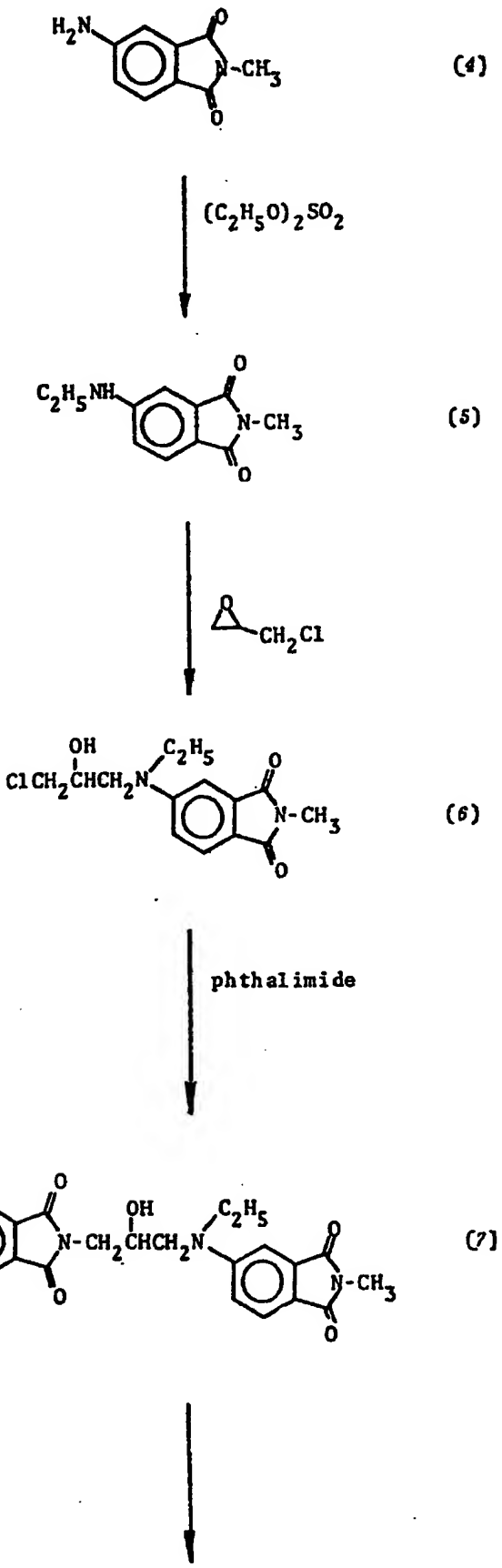
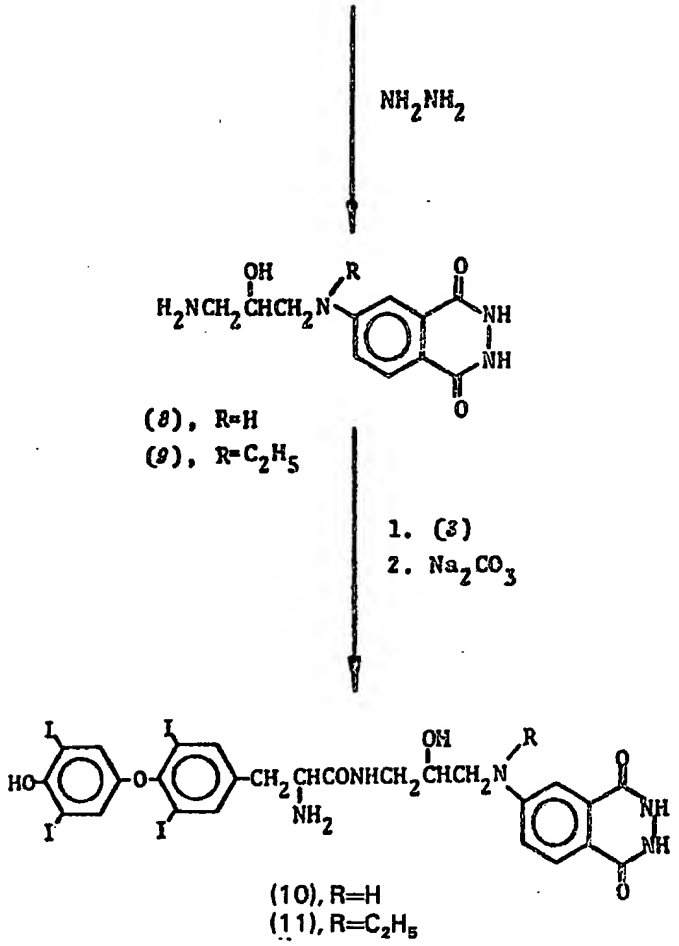


TABLE 6 (continued)



N-Trifluoroacetylthyroxinyl Ethyl Carbonic Anhydride (*3*).

A mixture of 0.17 ml of triethylamine and 1.05 g (1.2 mmol) of N-trifluoroacetylthyroxine (*2*) was dissolved in 20 ml of dry dimethylformamide at −10°C under anhydrous conditions [Knappe *et al, Biochem. Z. 338:* 599 (1963)]. To this was added a solution of 0.14 ml (1.2 mmol) of ethyl chloroformate in 2.9 ml of dry ether. After 30 minutes the precipitate of triethylammonium chloride was removed by filtration. The filtrate, now containing the anhydride (*3*), was used without isolation in the reaction described below to form labeled conjugate (*10*).

4-N-Ethylamino-N-methylphthalimide (*5*).

A mixture of 10 g (0.057 mol) of 4-amino-N-methylphthalimide (*4*) [Flitsch, *Chem. Ber. 94:* 2494 (1961)], 17.5 g (0.11 mol) of diethyl sulfate, and 100 ml of 2,2,2-trifluoroethanol was refluxed for one day. The reaction mixture was cooled, concentrated under reduced pressure, and the residue partitioned between 250 ml of ethyl acetate and 100 ml of saturated sodium bicarbonate solution containing 10 ml of triethylamine. The ethyl acetate phase was separated, washed with saturated aqueous sodium chloride solution, dried over anhydrous magnesium sulfate, filtered and evaporated. The residue was recrystallized twice from acetone-hexane and then from aqueous methanol to give 3.4 g (23% yield) of the phthalimide (*5*) as fine yellow crystals, m.p. 157°C.

Analysis: Calculated for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5 92; N, 13.71
Found: C, 64.00; H, 5.71; N, 13.37
NMR Spectrum (C_5D_5N): δ 0.5 (t, J = 7 Hz, 3H), 2.1 (s, 3H)

4-[N-(3-Chloro-2-hydroxypropyl)-N-ethylamino]-N-methylphthalimide (*6*).

A mixture of 3.1 g (15 mmol) of th phthallmide (*5*), 1.2 g (15 mmol) f 1-chloro-2,3-epoxypropane, and 30 ml of 2,2,2-trifluoroethanol was refluxed for 24 hours. At the end of this tim , another 1.2 g of 1-chloro-2,3-epoxypropane was added. After heating for an additional 24 hours, the reaction was cooled and evaporated and the residue chromatographed on 150 g of silica gel 60 (E. Merck, Darmstadt, West Germany), eluding with a 9:1 mixture (v:v) f carbon tetrachloride and

acetone. Fifteen ml fractions were collected. Fractions numbered 90 to 160 wer combined and evaporated to give 3 g of a yellow-red oil. The oil was crystallized from acetone-hexane and recrystallized twic from aqueous methanol to give 1 g (22% yield) of the phthalimide (*6*) as fin yellow needles, m.p. 123°C.

Analysis: Calculated for $C_{14}H_{17}ClN_2O_3$: C, 56.66; H, 5.78; N, 9.44
Found: C, 56.50; H, 5.93; N, 9.26
NMR Spectrum (C_5D_5N): δ 0.65 (t, 3H, J = 8 Hz), 2.6 (s, 3H)

4-[N-Ethyl-N-[2-hydroxy-3-(N-phthalimido)propyl]amino]-N-methylphthalimide (*7*).

A mixture of 25 g (0.08 mol) of the phthalimide (*6*), 23 g (0.13 mol) of potassium phthalimide, and 150 ml of dry dimethylformamide was refluxed for 36 hours. Removal of the solvent left a brown residue that was triturated with methanol to give 19 g of yellow solid. Recrystallization from aqueous acetic acid, then from aqueous methanol, gave 16 g (49% yield) of the *bis*-phthalimide (*7*) as a yellow solid, m.p. 158—160°C.

Analysis: Calculated for $C_{22}H_{21}N_3O_5$: C, 64.85; H, 5.20; N, 10.31
Found: C, 64.81; H, 4.97; N, 10.54
NMR Spectrum (d_6-DMSO): δ 1.2 (t, 3H, J = 6 Hz), 3.0 (s, 3H)

6-N-(3-Amino-2-hydroxypropyl)amino-2,3-dihydrophthalazine-1,4-dione (*8*).

This compound was prepared according to the method described both above relating to the biotin conjugate and in *Anal. Chem. 48:* 1933 (1976). As reported above, the efficiency of this amino-derivative (*8*) in the hematin catalyzed chemiluminescent reaction was 10% and the detection limit 20 pM.

6-[N-(3-Amino-2-hydroxypropyl)-N-ethylamino]-2,3-dihydrophthalazine-1,4-dione (*9*).

A mixture of 15 g (0.037 mol) of the *bis*-phthalimide (*7*), 60 ml of 95% hydrazine, and 300 ml of absolute ethanol was refluxed for 3 hours. The reaction was cooled, evaporated to dryness, and the crystalline residue dried at 40°C/0.05 mm Hg overnight. The residue was then dried at 120°C/0.05 mm Hg for 4 hours. The resulting solid was stirred for 3 hours in dilute hydrochloric acid and filtered. When the pH of the filtrate was adjusted to 7.0, a precipitate formed amounting to 4.6 g (46% yield) of the amino-phthalhydrazide (*9*), m.p. 207—210°C (decomposed). A small sample was recrystallized from H_2O to give white crystals, m.p. 208—211°C (decomposed).

Analysis: Calculated for $C_{13}H_{18}N_4O_3$: C, 56.10; H, 6.52; N, 20.13
Found: C, 55.61; H, 6.50; N, 20.35

The efficiency of the amino-derivative (*9*) and its detection limit were determined in the same manner as described above for the amino-derivative (*8*). The efficiency was formed to be 46% and the detection limit 5 pM.

6-N-[2-Hydroxy-3-(thyroxinylamido)propyl]amino-2,3-dihydrophthalazine-3,4-dione (*10*).

A suspension of 600 mg (2.4 mmol) of the amino-derivative (*8*) in 20 ml of dry dimethylformamide containing 1 ml of pyridine was stirred under argon for one hour. It was then drawn up into a syringe and added all at once to a −10°C solution of 1.2 mmoles of N-trifluoroacetylthyroxinyl thyl carbonic anhydride (*3*) in 20 ml of dimethylformamide. After stirring for 20 minutes at −10°C, the reaction was allowed to warm to room temperature and stirred overnight. Solvent was removed under high vacuum. The solid residue was stirred for 40 minutes in dilute hydrochloric acid, then filtered and dried under high vacuum to give 1.27 g of a free-flowing powder.

The trifluoroacetyl protecting group was removed by stirring 1.0 g of this powder for 5 hours in a methanol/H_2O solution. The pH of the solution was adjusted to 10.7 with solid sodium carbonate. The pH was reduced to 7.0 with hydrochloric acid and a white precipitate collected and dried. When dry this amounted to 700 mg (59% yield) of the labeled conjugate (*10*) as a yellowish-white powder, m.p. 235°C (decomposed).

Analysis: Calculated for $C_{26}H_{23}I_4N_5O_6$: C, 30.95; H, 2.30; N, 6.94
Found: C, 30.17; H, 2.33; N, 6.33

6-[N-Ethyl-N-[2-hydroxy-3-(thyroxinylamido)propyl]amino]-2,3-dihydrophthalazine-1,4-dione (*11*).

A solution of 1.06 g (1.2 mm l) of N-trifluoroacetylthyr xine (*2*) in 20 ml of dimethylformamide containing 0.17 ml f triethylamine was cooled to −10°C. To this was added 0.14 ml (1.2 mmol) of ethyl chloroformate. After 30 minutes at this temperatur the solution, now containing the mixed anhydride (*3*), was filtered to remove precipitated triethylamine hydrochloride and added to a suspension of 668 mg (2.4 mmol) of the amino-derivative (*9*) in 20 ml of dimethylformamide. After

stirring two days at room temperature, the solvent was rem ved under vacuum, and th residue washed with 10% hydrochld ric acid, collected by filtration and dried.

To remove the trifluoroac tyl bl cking group, the solid was dissolved in 50 ml of 0.1 M sodium carbnate (pH 10.5) to which was added a small amount of dimethylformamide to achieve soluti n. After one day at room temperature, it was evaporated to dryness. The residue was taken up in 30 ml of H_2O, and the pH adjusted to 7.2 with dilute hydrochloric acid. A solid precipitated that was collected and dried at 60°C under high vacuum to give 600 mg (50% yield) of the labeled conjugate (*11*) as white crystals, m.p. > 240°C (decomposed).

Analysis: Calculated for $C_{28}H_{27}I_4N_5O_6$: C, 32.42; H, 2.62; N, 6.75
Found: C, 29.81; H, 2.69; N, 5.45

B. Binding Assay for Thyroxine

Competitive binding reaction mixture (120 μl) were assembled in triplicate by combining the following reagents: 12 μl of 100 mM Tris-HCl (pH 8.8), 12 μl of 77 nM labeled conjugate (*11*) [labeled conjugate (*10*) could be used as well] in 10 mM Tris-HCl (pH 8.8), varying volumes of 40 nM thyroxine in the same buffer, 10 μl of a preparation of antibody to thyroxine in 5 mM phosphate buffer (pH 6.7), and a sufficient volume of H_2O to make a final volume of 120 μl. After a 1 hour incubation at room temperature, the free- and bound-species of the labeled conjugate were separated for each reaction mixture by applying a 100 μl aliquot to small columns (0.3 μl bed volume) of Sephadex (Registered Trade Mark) G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) previously washed with 10 mM Tris-HCl (pH 8.8). The bound-species of the labeled conjugate was eluted from the column with 0.5 ml of the same buffer leaving the free-species in the column.

An aliquot (115 μl) of each column effluent was added to 35 μl of 0.29 μM hematin (Sigma Chemical Co., St. Louis, Missouri USA) and 214 mM sodium hydroxide in a 6 x 50 mm test tube. Each tube was placed in the Dupont 760 Biometer and 10 μl of 90 μM hydrogen peroxide in 10 mM Tris-HCl (pH 7.4) were added. The resulting peak intensity of the light produced in each chemiluminescent reaction was recorded from the instrument reading and the results from the triplicate runs were averaged.

The relationship of the amount of thyroxine in the binding reaction to peak light intensity is shown in Table 6 below.

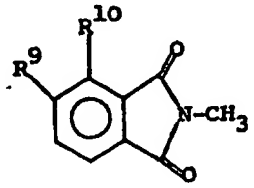
TABLE 6

volume of thyroxine solution added (μl)	peak light intensity
0	13.8
12	13.5
48	3.5

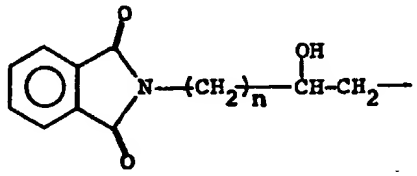
The results demonstrate that the labeled conjugate of the present invention is useful in binding assays for determining a ligand in a liquid medium.

CLAIMS

1. A compound of the formula:



wherein one of R^9 and R^{10} is hydrogen and the other is —$NR^{11}R^{12}$; R^{11} is hydrogen or straight chain alkyl containing 1—4 carbon atoms and R^{12} is



wherein n = 1 to 3.

2. A compound according to claim 1 wherein R^9 is —$NR^{11}R^{12}$.
3. A compound according to claim 1 or 2, wherein n = 1.
4. A compound according to any of claims 1 to 3, wherein R^{11} is hydrogen.
5. A compound according to any of claims 1 to 3, wherein R^{11} is ethyl.
6. 4-[3-(N-Phthalimido)-2-hydroxypropylamino]-N-methylphthalimide.
7. 4-[N-Ethyl-N-[2-hydroxy-3-(N-phthalimido)-propyl]amino]-N-methylphthalimide.

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